Quantifying Proteomic ICPL Samples by ESI Ion Trap MS

A. Kettani¹, U. Schweiger-Hufnagel², M. Behrens², and C. Baessmann²
¹Bruker Daltonics Inc., Billerica, Massachusetts, USA; ²Bruker Daltonik GmbH, Bremen, Germany

Quantification is increasingly important in proteomic research. One of the leading approaches is mass spectrometry combined to isotopic labeling. Besides the Stable Isotope Labeling with Amino acids in Cell culture (SILAC) which is restricted to bacteria and selected higher organisms, a highly relevant way is the modification of specific amino acids with labeled tags (SILE; Stable Isotopic Label Experiments). For our experiments we used ICPL (Isotope Coded Protein Label), and the major advantages of our approach are shown on the poster: (1) the use of ICPL which can be perfectly combined to proceeding protein separation techniques, and (2) a sophisticated technique to detect even low-abundant SILE pairs for automated fragmentation.

Two samples were prepared containing proteins with various concentrations (standard protein mixtures and bacterial cell lysate). Both samples were labeled using the ICPL kit. In approach (1) both samples were combined, the proteins were separated by gel electrophoresis, excised and digested. In approach (2) the proteins were digested without protein separation. Both peptide samples were separated by nano LC, analysed by ESI mass spectrometry using the new SILE pair detection technique and quantified.

SILE protein quantification results are shown from both unseparated and separated protein samples analysed on an ESI ion trap. The high result quality shown here benefits from several features: (1) a label which modifies amino acids on intact proteins allowing to separate them, which is most useful for complex samples, (2) an ultra-fast and highly-sensitive ion trap instrument, (3) the option to automatically detect SILE pairs during data acquisition and to fragment them instantaneously, which increases the number of pairs used for quantification and therefore is of particular importance for complex peptide mixtures, (4) an advanced outlier treatment for quantification, and (5) the extensive grade of automation for the whole procedure using a project-based result database software.

Long-term Archiving of Proteomics Samples on Disposable MALDI Targets

C. Luebbert¹, C. Ziegmann², D. Suckau¹, and M. Schuerenberg¹
¹Bruker Daltonik GmbH, Bremen, Germany; ²Eppendorf Polymere GmbH, Oldenburg, Germany

Archiving of MALDI sample preparations is an important issue not only with regard to classical long-term storage triggered by legal or GLP demands. It becomes relevant, whenever there is a significant time lag between sample preparation and MS analysis and whenever selected samples are revisited under different aspects or to verify results. Any kind of long-term archiving requires a cost-effective reliable platform technology and dedicated storage conditions. Here, a disposable plastic MALDI target was used for archiving proteomics samples. Archiving is valuable for protein digest analysis after 2D gel separation as well as LC-MALDI analyses, as the success of an analysis—or the need for further data acquisitions—may only be clear to the scientist well after the primary data acquisition took place.

250 amol and 5 fmol tryptic BSA digest were prepared on the HCCA matrix anchors of a number of prespotted AnchorChip targets (Bruker Daltonics). Right after preparation a first set of MS and MS/MS reference data was acquired on a MALDI-TOF/TOF. Then the targets were sealed in plastic bags with different gas fillings and were stored at different temperatures. We revisited the samples monthly under well defined experimental conditions in order to check their mass spectrometric performance (sequence coverage, intensity coverage, number of matching peptides) after up to 12 months of storage time.

After that period of time, the same spectra quality decrease was observed for samples stored at room temperature under nitrogen. Although S/N slightly decreased with time, safe protein identification was achieved even at the 250 amol level. The sequence coverage remained unchanged at about 40%.

Real-life 2DGE spots were investigated: result from ca. 80 in gel digests after 2DGE shows, that the mascot protein scores are basically unchanged 1 month after sample preparation on the MALDI target.
Examination and Identification of Signalling Components Involved in Cellular Response to Radiation by Using a Phosphoproteomic Approach

S. Ståhl¹, K. Viktorsson², J. Lennqvist³, B. Stenerlöw³, R. Lewensohn², and J. Lehtio¹

¹Karolinska Biomic Centre Karolinska Institute, Stockholm, Sweden; ²Karolinska Institute, Stockholm, Sweden; ³Uppsala University, Uppsala, Sweden

Resistance to radiotherapy is a major clinical problem among cancer in general and in lung cancer in particular. Radiation given as anticancer regimes causes several types of DNA damages such as single-and/or double DNA strand breaks (DNA dsbs) as well as base damages which can be converted into DNA dsbs upon DNA repair. Radiation in part mediate its effects by triggering apoptotic cell death hence non-functional apoptotic signalling pathway may in part account for resistance towards radiation. Mitochondria is of crucial importance for the propagation of proapoptotic signalling from the initial DNA damage. However still there are links missing in the signalling cascades which mediate the proapoptotic effects. It is nevertheless likely that several of these signalling proteins are phosphorylated and hence identifying and quantifying phosphorylated proteins/peptides globally in cells will most likely results in discovery of proteins which can be used as novel radiosensitizers.

In an on going study, we will therefore assess if differences in signal transduction i.e., protein phosphorylations are observed after exposure to different radiation qualities in NSCLC cell lines. Currently, phosphorylation signalling events have mainly been assessed in a non-global way using kinase assays or phospho-specific antibodies in western blot. Here we analyze global and quantitative changes in protein phosphorylation in complex samples using multi dimensional enrichment of phosphorylated peptides, optimal sample conditions in combination with high technology proteomic methods such as iTRAQ and suitable mass spectrometry. This will be used in combination with standard assays in apoptotic signalling.

The results of this study will be presented and further discussed.

Proteome Analysis of Low Molecular Weight Proteins in Halobacterium salinarum


Max Planck Institute of Biochemistry, Martinsried, Germany

Systematic investigation of low molecular weight proteins (LMW, below 20 kDa) in the archaeon H. salinarum resulted in the identification of 383 proteins, which represent 35% of the theoretical proteome in that size range. The improved identification ratio for LMW proteins was achieved by optimisation of common protocols for protein analysis. LMW proteins could be rapidly and effectively enriched by filter membrane centrifugation followed by tricine SDS-PAGE. Standard gel staining and in-gel digestion protocols proved to cause a significant loss of LMW proteins. Protocols were improved to allow full recovery of LMW proteins. In addition to a series of technical problems, small proteins may show low gene expression levels as concluded from a low average codon adaptation index.
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Comparison of High-throughput Serum Peptide Capture Methods for Peptide Profiling by MALDI-TOF Mass Spectrometry
C. Jimenez1, Z. El Filali1, E. Marchiorit2, K. W. Li3, K. Hoekman1, and G. Giaccone1
1VU University Medical Center, Amsterdam, The Netherlands; 2Vrije Universiteit, Amsterdam, The Netherlands

Serum peptide profiling by mass spectrometry is an emerging approach for disease diagnosis and biomarker discovery. A magnetic bead-based method for off-line serum peptide capture coupled to MALDI-TOF mass spectrometry has been introduced recently. However the reagents are not available to the general scientific community.

Here, we optimized and compared two high-throughput methods for serum peptide sample processing: 1. novel C18 magnetic bead (Dyna-beads® RPC 18) in conjunction with a high-throughput magnetic particle processor, the KingFisher® 96 (ThermoElectron Corp.) and 2. solid-phase extraction in 96-well plates (Universal resin, Empore 3M) and vacuum extraction. We investigated peptide binding and elution conditions. The methods are evaluated in terms of relative peaks counts and reproducibility of ion intensities in control serum. Both methods allow for reproducible detection of up to a few hundred serum peptides in the mass range of 0.8–10 kD. Using the extraction disks, we have identified peptides sensitive to differences in serum sample handling (clotting time). Disease- and drug-response profiling studies are on-going.

In summary, the DynaBead- and the universal resin extraction disk-based serum sample processing protocols reported here are reproducible and robust and can be used for serum peptide profiling by anyone in possession of a MALDI-TOF instrument. In conjunction with the King-Fisher® 96 or a vacuum extraction unit, the whole serum peptide capture procedure is high-throughput (~20 min per isolation of 96 samples in parallel), thereby facilitating large-scale disease profiling studies.

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GFS Version 2, A Comprehensive Software System for Protein Identification Using Unannotated Genome Sequence Data
J. Khatun1, J. Miller1, D. Yang1, M. S. Wisz2, R. Balasubramaniyan1, and M. C. Giddings1
1The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA; 2Emergent, Inc, New York, New York, USA

Most protein identification software relies upon databases of known genes and/or proteins against which mass spectrometry (MS) data is matched, which limits the accuracy of identification to that of the underlying genome annotations. This can be particularly stifling for cases such as alternative-siliged genes, where alternative exons are often missed, or for genes that have been sequenced but not annotated yet. We developed Genome-based peptide Fingerprint Scanning (GFS) for identifying proteins by mapping MS data directly to raw, unannotated genome sequence, bypassing the reliance on genome annotation for protein identification.

The newly released version 2 of GFS represents significant advances in many areas, and is now freely available open source software that runs on multiple platforms including Windows, Linux, and Mac OS X. Performing identification for both MS and MS/MS samples, GFS is designed for efficient distributed operation on a compute cluster, and can batch-process samples. We have implemented scoring using the expectation value method, and GFS can now perform searches against annotated genes in addition to raw genome sequence, to facilitate comparison of the results. We have developed several new machine-learning based scoring systems that will be further detailed in separate presentations. We present an overview of GFS, focusing on the new features, and its application for genomic annotation. GFS is available for download from http://gfs.unc.edu, and a limited version is available for web-based usage at the same site.

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Estrogen Receptors in Seminal Vesicle and Prostate; Do They Play a Role in Male Fertility?
T. Schwend, O. Imamov, C. Williams, and J.-Å. Gustafsson
Karolinska Institute, Huddinge, Sweden

Seminal vesicle and prostate are secretory organs which play an important role in male fertility. The former secretes clot-forming proteins to the semen which immobilize the sperm to a large extent. Clot-forming and immobilization appears to be important for sperm maturation (capacitation). The prostate secretes serine proteases to the semen which degrade the clot-forming proteins (liquefaction) and thereby liberate the sperm. Expression of clot-forming proteins and serine proteases is regulated by the androgen receptor, a member of the nuclear receptor family. In addition to androgen receptor, seminal vesicle and prostate gland are rich in estrogen receptor α (ERα) and the later in ERβ. Recent studies of ERα and ERβ knock out mice showed that both receptors play an important role in the early development and branching morphogenesis of the prostate. However, how and to which extent these receptors contribute to male fertility remains unclear.

By using isotope-coded protein labeling, mass spectrometry and immunohistochemistry we show that ERα and ERβ regulated the level of serine protease inhibitors in the murine prostate and seminal vesicle. Serine protease inhibitors are secreted proteins which oppose the effect of the prostatic serine proteases and consequently influence liquefaction time. Therefore ERα and ERβ may play an important role in male fertility.
Halobacterium species predominantly occurs by the tricarboxylic acid (TCA) cycle, while under anaerobic conditions, ATP synthesis in Halobacterium species predominantly occurs by the tricarboxylic acid (TCA) cycle, while under anaerobic conditions, ATP synthesis in Halobacterium species predominantly occurs by the tricarboxylic acid (TCA) cycle, while under anaerobic conditions, ATP synthesis in Halobacterium species predominantly occurs by the tricarboxylic acid (TCA) cycle, while under anaerobic conditions, ATP synthesis in.
The DFS Tool for Dissemination and Annotation of Proteomics Data

J. Falkner and P. C. Andrews

University of Michigan, Ann Arbor, Michigan, USA

Dissemination of raw and processed proteomics data is a major challenge for proteomics and recent efforts to make datasets public are responses to quality control needs for accurate interpretation, the public nature of much of the funding for proteomics research, and the need for improved algorithms requiring access to datasets. Only 1.3 terabytes of proteomics data is currently (May, 2006) easily accessible online, underscoring the need for improved methods for sharing and disseminating data.

The ProteomeCommons.org system for dissemination and annotation addresses these problems through a robust and secure grid-like storage system with a simple user interface. Advantages of this system include: file format independence, ability to manage both very large files and large numbers of files, support for incorporating any data annotation scheme, ability to securely control access to data, ability to retract data files, ability to search across all data files, file replication, and distributed downloads. Setup and maintenance requirements are minimal, allowing individual laboratories to publish their own data files. Distributed downloads and background file compression are supported for large data files. Support for modern public/private key encryption enables the concept of Authorities, allowing a range of functions, including peer review, retraction of data, annotation of datasets, limited lifespans of published data, etc. An Authority can be any entity with a commercial RSA or DSA key pair (a common component of a website) or an independently generated key pair, including research institutions, journals, individual laboratories, or companies. This also allows service level agreements among Authorities and service providers. The software for the system complies with open source standards, allowing for free community access, development and customization of code for specific applications. The Dissemination System currently resides on 26 servers with aggregate storage space of approximately 50 terabytes.

Peptide and Protein Analysis by MALDI-TOF MS Using Magnetic Bead Based Metal Ion Affinity Chromatography

T. Elssner1, K. Blaesing2, G. Shi3, and M. Kostrzewa1

1Bruker Daltonik, Leipzig, Germany; 2Hochschule Anhalt (FH), Köthen, Germany; 3Bruker Daltonics, Billerica, Massachusetts, USA

Immobilized metal ion affinity chromatography (IMAC) is a widely used separation method for peptides and proteins on the basis of their affinity to metal ions which have been immobilized by chelation. IMAC is based on specific interaction between certain amino acid side chains or posttranslational modifications as phosphorylation and immobilized metal ions, such as Ni2+, Cu2+, Co2+, Fe3+, Ga3+, or Al3+. IMAC is an excellent method for purification of histidine-tagged recombinant proteins as well as for enrichment of phosphorylated peptides from complex peptide mixtures.

Here, we present the enrichment of diverse peptides and proteins using magnetic particles chargeable with various metal ions and their subsequent analysis by MALDI-TOF MS. Magnetic beads carrying non-charged chelators were loaded with different metal ions (Ni2+, Fe3+, Cu2+, Co2+, Al3+, Zr2+, Mn2+).

Synthetic phosphopeptides and a digest of phosphoproteins were used as model systems to demonstrate the enrichment on the surface of magnetic beads carrying immobilized Fe3+. Histidine-tagged proteins were captured by usage of immobilized Ni2+. Additionally, the various IMAC beads carrying different immobilized metal ions were used for generation of profiles from human sera.

In any case, after elution from the beads peptides/proteins enriched were spotted directly onto a Prespotted AnchorChip (with α-cyano 4-hydroxy cinnamic acid) target or alternatively onto a standard AnchorChip target and subsequent analyzed with a MALDI-TOF mass spectrometer. Enrichment of peptides/proteins by IMAC on the surface of magnetic beads followed by MALDI-TOF MS analysis enables different application fields as analysis of posttranslational modifications and recombinant proteins or prefractonation of clinical samples, particularly serum for proteomic profiling studies. According to question of interest the magnetic beads can be charged with metal ions of choice.
Rapid and Time-controlled Protein Digest by Trypsin-coated Magnetic Particles Prior to Mass Spectrometric Analysis

S. Tigrett¹, M. Canton¹, T. Elssner², M. Kostrzewa², and K. Pacaud-Mercier¹
¹Agro-Bio, LaFerte StAubin, France; ²Bruker Daltonik, Leipzig, Germany

Protein enzymatic degradation followed by HPLC separation of peptides has been the choice method for years to analyze protein sequences. However these methods are time-consuming and expensive, therefore ill adapted to high throughput protein studies. Mass spectrometry now allows the rapid analysis of both peptides and proteins, yielding great amounts of information when combined to enzymatic digestion. Protein digestion is carried out after purification steps, either in gel on the separated proteins or in solution on the purified fraction. Both in-gel and in-solution digestions present a few draw-backs: presence of trypsin auto-digestion peaks, lack of digestion kinetic control, higher protein quantities needed. We propose here a novel digestion method, which avoids these inconveniences and dramatically reduces manipulation time.

Therefore, modified trypsin has been bound to microparticles with a paramagnetic core by a covalent attachment. These trypsin beads were used in classical in-gel or in-solution digestion protocols, replacing free trypsin. The digestion peptides obtained were directly analyzed by Autoflex Tof-Tof (Bruker) mass spectrometry. For example, 100 ng of digested bovine serum albumin yielded 45% sequence recovery with 31 peptides in only 15 minutes and showed 23% sequence recovery with 16 peptides in just one minute. Furthermore, a 5-minute digestion produced 12% sequence recovery with 5 peptides for carbonic anhydrase (29.1 kDa) and haptoglobin’s (345 kDa) two chains gave respectively 31% and 28% sequence recovery with 16 and 16 peptides.

A novel workflow strategy consisting of an upstream magnetic bead associated chromatography (e.g. on hydrophobic beads) followed by a time-controlled digest of the eluate using Trypsin-coated magnetic particles was established. The digest was subsequently analyzed by MALDI-TOF mass spectrometry in the reflector mode. Reproducibility of mass spectra generated could be demonstrated. Further combinations with other upstream magnetic bead based enrichment strategies are in progress.

Evaluation of Different Interpretation Strategies to Discover PTM in MS/MS Peptide Fragmentation Data

D. C. Chamrad¹, G. Körtting¹, K. Fantom², A. West², K. Schneider², U. Schweiger-Hufnagel², H. Thiele³, and M. Blüggel¹
¹Protagen AG, Dortmund, Germany; ²GlaxoSmithKline, Harlow, Great Britain; ³Bruker Daltonik GmbH, Bremen, Germany

Nowadays high throughput identification of peptides and proteins from large MS/MS datasets is a standard procedure within proteomics projects. However, usually only a small portion of spectra can be explained and a great challenge is the detection of post translational modification (PTM) which can be seen as a needle in the haystack problem.

To address the needs for more exact primary structure definition and PTM detection, promising software has become available performing a second pass search, which is restricted to already identified proteins. Here we evaluate those tools and compare the performance with standard database search software.

LC-MS/MS data was acquired from tryptic digested MAP-Kinase 12 and MAP-Kinase 13 proteins. This dataset was analysed with the second pass search tools Mascot (-error tolerant search) and our in house developed software PTM-Explorer.

Using second pass search strategies various modifications were detected, namely phosphorylation, methylation, pyro-glutamate formation, methionine oxidation and carbamido-methylation. In case of PTM-Explorer even five different phosphorylation sites were detected, some have been already described in Swissprot. There were also a significant number of one side non-specifically cleaved peptides increasing overall sequence coverage. Additionally spectra were clearly assigned to MAP-Kinase sequence, but could not be matched to a theoretical full precursor ion mass. Compared to standard database searches the amount of explained spectra was nearly doubled.

As manual spectra evaluation is time critical, and current automation allows generation of a tremendous amount of spectra in short time, MS interpretation tools are challenged to cut down manual analysis time significantly. Therefore we also evaluated the efficiency of result presentation and the required manual interpretation time. PTM-Explorer result visualisation was most efficient as it clusters the spectra according to amino acids, provides different views on the dataset and can filter or sort results.
Fully Automated Off-line Multidimensional LC Methods in Proteomics

R. van Ling, E.-J. Sneekes, B. Dolman, and R. Swart
LC Packings–Dionex, Amsterdam, The Netherlands

Multidimensional liquid chromatography (MDLC) coupled to mass spectrometry is a valuable strategy for bottom-up or top-down workflows in proteomics. A large number of different MDLC approaches has been described for the separation of intact proteins and peptides.

Off-line MDLC techniques have several advantages over on-line approaches: i) higher flexibility with respect to column dimensions and mobile phase selection, ii) easier method development and iii) the ability to perform re-analysis of the fractionated effluent.

Here we present a fully automated method for off-line MDLC of peptides and proteins. The method allows for combination of different column dimensions and chemistries to optimize the MDLC separation. The effluent from the first dimension column is re-directed to an 8-port injection valve and fractionated through the injection needle in a well plate or sample vials.

The second dimension separation comprises a column switching configuration for on-line desalting, sample concentration and direct interfacing to MS. Alternatively for protein separations a second fractionation step can be implemented after the RP separation to allow for proteolytic digestion.

Methods have been developed for 2D-LC of peptides and proteins using ion-exchange and reversed phase chromatography. Fractionation of the ion-exchange separation and subsequent injection of the fractions is performed automatically onto capillary PS-DVB monolithic columns. The fast mass transfer kinetics of the monolithic columns allow fast gradient separations and a reduction of the total analysis time of the 2D-LC method. Interfacing the 2D-LC separation with tandem MS sequencing of peptides showed that peptides eluted typically in one or two SCX fractions. In addition the system shows good precision performance for injection and fractionation of samples.

Protein Identification and Result Processing in Multi-workflow Studies

P. Hufnagel¹, S. Hahner¹, M. Lubeck¹, G. Körting², J. Glandorf¹, U. Schweiger-Hufnagel¹, W. Jabs¹, and D. Suckau¹
¹Bruker Daltonics, Bremen, Germany; ²Protagen AG, Dortmund, Germany

Current shotgun proteomics approaches fall short in reproducibly providing protein identification and quantification on greater number of proteins. Therefore, biological and technical replicates are mandatory as well as the combination of the mass spectral data from various workflows (gels, 1D, 2D-LC) various instruments (TOF/TOF, trap, qTOF or FTMS) and search engines (Mascot, Phenix, Sequest). Here we describe a database-driven study that combined multiple workflows, mass spectrometers and 3 search engines with decoy database protein identification.

The sample was a trypsin digested lysate of 10,000 cells of a human colorectal cancer cell line. Two data dependent LC-MALDI-TOF/TOF runs and a 2D-LC-ESI-trap runs on capillary and nano-LC columns were acquired and submitted to the proteomics database software package ProteinScape. The combined MALDI data and the ESI data were searched using Mascot (Matrix Science), ProteinSolver (Bruker and Protagen) and Sequest (Thermo) against a decoy database generated from IPI-human in order to obtain a gross protein list across all workflows and search engines at a defined maximal false positive rate for protein identification of 5%. The reproprocessing of the MS data followed the HBPP guidelines (forum.hbpp.org). The initial separate searches from the 2 datasets generated 6 independent protein lists, which were merged into a single list and all redundant protein assignments were removed from the single result list using the ProteinMerger algorithm in ProteinScape.

Initial evaluation of the generated data provided for the identification of ~1100 proteins derived from the 2D-LC-ESI approach and ~800 proteins from the LC-MALDI approach with a decoy estimated false positive rate of 5%.
The halophilic archaean *Halobacterium salinarum* lives in habitats with sodium chloride concentrations near saturation. It harbors a specialized signal transduction network connected to bioenergetics for finding optimal growth conditions which has been well-characterized over the past decades. This makes *H. salinarum* an attractive subject for modelling cellular processes with systems biological approaches.

A prerequisite for modelling cellular modules is to know the components of such modules, for example proteins, and how these components are interconnected, e.g. which proteins interact. Whereas the analysis of the halobacterial proteome has made large progress, the analysis of protein-protein interactions (PPI) in this organism is still at the beginning. Due to the halophilic nature of the proteins most of the established methods for PPI analysis (for example the yeast two-hybrid system) are not applicable.

We are using affinity purification of protein complexes combined with mass spectrometry to identify the interaction partners of a protein. Since the high-salt adapted proteins from *H. salinarum* require multimolar salt concentrations to preserve their conformation and their interactions, these salt levels have to be kept during the whole purification process. Therefore we express full-length proteins fused to a cellulose-binding domain (CBD), that binds to cellulose in a salt-insensitive manner directly in *H. salinarum* and purify them under native conditions.

After purification, the protein complexes are resolved by 1D gel electrophoresis and its components identified by liquid chromatography/tandem mass spectrometry (LC/MS/MS). As the one step purification leads to quite high levels of background proteins, stable isotope labeling by amino acids in cell culture (SILAC) is applied to discriminate the true interaction partners from background. Quantification of differentially labeled proteins is done with the tool ASAPRatio included in the Trans-Proteomic Pipeline.

### Analysis of Protein-Protein Interactions in *Halobacterium salinarum*

**M. Schlesner, H. Besir, B. Scheffer, M. Aivaliotis, and D. Oesterhelt**

Max Planck Institute of Biochemistry, Martinsried, Germany

Two-dimensional gel electrophoresis (2-DGE) is a powerful analytical method for high resolution separation of complex mixtures of gene products which remains nowadays a key tool for proteomics. Advancement in proteomic research is closely linked either to the improvement of current analytical techniques or to the emergence of new technologies. A good example is the huge step forward in protein identification and characterization generated by the recent breakthrough in mass spectrometry (MS) and bioinformatic. Considering 2-DGE technique, if considerable work have been achieved to improve isoelectric focussing step, no significant improvements have been reported for the second dimension using standard poly(acrylamide) gels, which are suffering from severe limitations related to a lack of resolution and poor recovery of peptide fragments for further MS analysis.

In this context, we developed a new gel matrix, named EDGE, optimised for high performance 2-D electrophoresis and based on proprietary NAT (N-acryloyl-tris-(hydroxymethyl)aminomethane) monomer, a hydroxylated acrylamide derivative. The unique gel composition led to a specific polymer network arrangement exhibiting outstanding properties for the recovery of tryptic fragments after in-gel digestion. Indeed, when we compared to commercially available poly(acrylamide) gels, NAT based gels provided MS spectra with more numerous peptides and higher peak intensities. Sequence coverage reached high values so that unambiguous protein identification was possible. Spectacular results were obtained with low expressed proteins that could not be identified with poly(acrylamide) gels. Such performances were obtained with no compromise on others key electrophoretic parameters. Indeed, we will show that NAT based gels also offer excellent and consistent spot resolution in the whole protein size range, strong mechanical properties and are fully compatible with Coomassie, silver and fluorescent staining methods.

### X!Tandem Discriminant Functions Utilizing High-Mass-Accuracy Precursor Ion Data

**L. Everett, I. J. Musselman, and S. R. Master**

University of Pennsylvania, Philadelphia, Pennsylvania, USA

False positive estimation using PeptideProphet has become a standard approach for post-processing of peptide identification results. However, the limited availability of appropriate discriminant scores for search engines other than Sequest and Mascot has hampered the incorporation of open-source search engines such as X!Tandem into this analysis pipeline. Further, discriminant scores have typically been derived from low mass-accuracy instruments rather than FTICR- or Orbitrap-based mass spectrometers capable of accurate precursor mass measurement and unambiguous charge state determination. In order to address these limitations, we have generated a standard mix consisting of a tryptic digest of 12 known proteins at a variety of relative molar concentrations and have repeatedly profiled this mixture on an LTQ-Orbitrap. In addition to this training mix, a test mix with altered molar ratios has been generated and analyzed. These data have been used to derive and validate discriminant functions for PeptideProphet that incorporate X!Tandem values for expectation value, hyperscore, next score, and mass error. Of note, the distribution of false positive discriminant scores is well-modeled by a Gaussian distribution, and the true positives are sufficiently close to a gamma distribution in the +2 and +3 charge states.

### New Generation of Gels for 2-D Electrophoresis Providing High Protein Identification Scores

**F. Montagne¹, J.-L. Roux-dit-Buisson¹, F. Guérard², O. Guerre², and C. Rolando²**

¹Elchrom Scientific, Cham, Switzerland; ²Université des Sciences et Technologies de Lille, Lille, France

Two-dimensional gel electrophoresis (2-DGE) is a powerful analytical method for high resolution separation of complex mixtures of gene products which remains nowadays a key tool for proteomics. Advancement in proteomic research is closely linked either to the improvement of current analytical techniques or to the emergence of new technologies. A good example is the huge step forward in protein identification and characterization generated by the recent breakthrough in mass spectrometry (MS) and bioinformatic. Considering 2-DGE technique, if considerable work have been achieved to improve isoelectric focussing step, no significant improvements have been reported for the second dimension using standard poly(acrylamide) gels, which are suffering from severe limitations related to a lack of resolution and poor recovery of peptide fragments for further MS analysis.

In this context, we developed a new gel matrix, named EDGE, optimised for high performance 2-D electrophoresis and based on proprietary NAT (N-acryloyl-tris-(hydroxymethyl)aminomethane) monomer, a hydroxylated acrylamide derivative. The unique gel composition led to a specific polymer network arrangement exhibiting outstanding properties for the recovery of tryptic fragments after in-gel digestion. Indeed, when we compared to commercially available poly(acrylamide) gels, NAT based gels provided MS spectra with more numerous peptides and higher peak intensities. Sequence coverage reached high values so that unambiguous protein identification was possible. Spectacular results were obtained with low expressed proteins that could not be identified with poly(acrylamide) gels. Such performances were obtained with no compromise on others key electrophoretic parameters. Indeed, we will show that NAT based gels also offer excellent and consistent spot resolution in the whole protein size range, strong mechanical properties and are fully compatible with Coomassie, silver and fluorescent staining methods.
Two-Dimensional Liquid Chromatography Analysis Software; Maximizing the Potential of a Powerful Proteomic Tool

S. T. Elliott¹, A. Olsson², S. Sheng¹, K. R. Boheler³, O. Forsstrom-Olsson², and J. E. Van Eyk¹

¹Johns Hopkins University, Baltimore, Maryland, USA; ²Ludesi AB, Lund, Sweden; ³National Institute on Aging, Baltimore, Maryland, USA

Multi-dimensional whole-protein liquid chromatography (LC) is an enormously powerful tool for proteome characterization. LC instruments can be stacked to provide multi-level separation of complex biological samples, based on multiple physical protein properties. The ProteomeLab PF 2D (Beckman Coulter, CA, USA) separates by isoelectric point on a pH-based chromatofocusing column and collects samples into 96 well plates for automated second dimension separation by automated second dimension separation by hydrophobicity using advanced non-porous chromatography. Using these units on murine embryonic stem cells, we have identified over 1000 non-redundant proteins with over 400 potential post-translational modifications.

However, quantitative comparisons of the subtle proteome differences between the examined embryonic stem cell lines required software beyond what was currently available. To meet this informatic demand, Ludesi AB is developing a software package specifically designed to organize and compare multiple 1D- or 2DLC runs. This software aligns and matches peaks from liquid chromatography profiles, allowing peak tracking from adjacent profiles and comparisons between samples or groups of samples. Peak quantification allows normalization, baseline correction and with 2DLC, integration of peaks from adjacent profiles. Using this software with our PF2D analysis of R1 and D3 mouse embryonic stem cells resulted in the identification of 12 regions of differentially regulated proteins that were confirmed by iTRAQ experiments and 1D gel electrophoresis.

Human Plasma Proteomics Based on Modified 2-D Gels with Higher In-Gel Digestion Recovery and Outstanding Protein Identification Scores

O. Guerre¹, F. Guérard¹, J.-L. Roux², F. Montagne², and C. Rolando¹

¹Université des Sciences et Technologies de Lille, Lille, France; ²Elchrom Scientific, Cham, Switzerland

Gel electrophoresis remains the principal tool for separation and identification of the complex mixture of proteins found in human plasma. To this purpose, so-called PAGE (PolyAcrylamide Gel Electrophoresis) is used for many years despite various limitations and in particular (i) a lack of resolution leading to spot diffusion or overlapping and (ii) a poor sequence coverage during tryptic fragment identification after in-gel digestion and mass spectroscopy analysis. In this context, we introduce a new gel technology for 2-D electrophoresis, named EDGE, especially developed to overcome these limitations. These new gels are based on proprietary NAT (N-acryloyl-tris-(hydroxymethyl)amino-methane) monomer, a hydroxylated acrylamide derivative. Various formulations have been evaluated on (1D) and (2D) gels in order to assess their properties. First, we will show that the gels are fully compatible with commonly used staining methods including Coomassie, silver and fluorescence. For example, detection sensitivity is found to be as little as 1 ng of proteins with Sypro Ruby™ staining.

Then, tests on human plasma depleted of albumin have been performed to evaluate the proteomics performance on this news gels. We found that the resolution is superior to the other types of gels, with spot shapes better defined (less lagging or overlap), and a consistent behaviour in the high as well as low molecular weight regions.

Finally, the most striking difference concerns the database identification of tryptic fragments after in-gel digestion and MALDI-TOF mass spectrometry analysis. Twice more spots were identified compared to the classical poly(acrylamide gels) and sequence coverage is by far better especially for low abundant proteins for which the sequence coverage is most often doubled.
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Multivariate Analysis Resolving Label-free Differential Mass Spectrometric Data

R. T. Nilsson¹, M. Bylesjö², V. Srivastava¹, and G. Wingsle¹

¹Umeå Plant Science Centre, Umeå, Sweden; ²University of Umeå, Umeå, Sweden

Proteome alterations can be monitored in detail on peptide level by mass spectrometry. The major obstacle to measure these differences is the dynamic range of protein abundance. In our approach we focus on identification and characterization of the sub-proteome of ionically bound cell wall proteins of stem tissue from Populus. A narrow pI fractionations of peptides obtained by capillary isoelectric focusing has been performed. Optimization of sample extraction, data processing and evaluation of results are supported by experiential design and multivariate methods. Limiting artifacts caused by sample handling and native modifications are improved to gain interpretation of the biological dynamics. Physicochemical properties of the peptides and their behavior in the last step of analysis (RP-nanoHPLC mass spectrometry) are used to validate the result.

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High-throughput Confocal Subcellular Mapping for Antibody-based Proteomics

L. Barbe¹, E. Lundberg¹, H. Brismar², M. Uhlen¹, and H. Andersson¹

¹Biotechnology, KTH, Stockholm, Sweden; ²Cell Physics. KTH, Stockholm, Sweden

To better understand the proteins function knowledge of the subcellular location is critical. Currently, fluorescence microscopy is the most suitable method for proteome-wide determination of subcellular location. This project, part of the Human Protein Atlas (www.proteinatlas.org) which is building an antibody-based atlas for normal and cancer tissues, is focusing on subcellular protein localization using fixed human cell lines and immunofluorescence techniques.

Immunostaining is performed on 12 cell lines of different phenotypes using automated liquid handler. Cells cultured in 96 well glass bottom plates are immunolabelled using in-house generated monospecific antibodies. Different probes specifically targeting main organelles (nucleus, mitochondria, endoplasmic reticulum, etc.) are used to identify in which subcellular compartment proteins of interest are expressed. A modified Zeiss LSM 510 confocal microscope enables automatic generation of multi-color 3D stacks in 96 well plates. The next step of this workflow is image analysis and data generation. Spatial colocalization between the antibody of interest and organelle probes is used to quantify overlapping regions from the different channels. Different colocalization algorithms (Pearson, Costes, etc.) are implemented to routinely generate correlation coefficients enabling systematic classification of antibodies into subcellular categories.

Preliminary results show that this method is robust and fast enough to generate high content information for large scale proteomics projects.

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Proteomic Profiling Studies in Glaucoma Using SeldiTOF and ClinProt Magnetic Beads

F. H. Grus, L. Hornberger, N. Wiegel, S. Berneiser, U. Thiel, and N. Pfeiffer

Dept. of Ophthalmology, Mainz, Germany

Glaucoma is one of the leading causes for blindness in the world. The glaucoma disease is characterized by a progressive loss of retinal ganglion cells. The aim of this study was to search for serum biomarkers for glaucoma using the SELDI ProteinChip (Ciphergen, USA) Technology and the ClinProt magnetic bead approach in combination with Maldi-TOFTOF (Bruker, Germany).

Sera of patients (n = 150, healthy volunteers (CTRL), primary-open-angle-glaucoma (POAG), ocular-hypertension (OHT), and normal-tension-glaucoma (NTG)) were fractionated by anion exchange resin and subsequently analyzed on three different ProteinChip arrays. Proteins were detected by surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). Proteins were fractionated prior to Maldi-TOFTOF analysis using C3, C8, C18, and WCX magnetic beads. The Maldi spectra were acquired using a Bruker Ultraflex MALDI TOFTOF, Identification of biomarkers was performed by enrichment of the proteins/peptides by SDS-PAGE or Nano-LC, tryptic digestion and Maldi-TOFTOF analysis.

Complex protein patterns were found in all patients. More than 4000 protein clusters were identified across the different chip surfaces and magnetic beads, fractions, and laser settings. Multivariate analysis of discriminace can test for statistical differences between the subgroups using the entire complex staining pattern for the calculation. The method successfully found a significant difference between all subgroups.

From these more than 4000 proteins, a panel of 8 potential biomarkers could be selected using a combination of multivariate analysis of discrimination, artificial neural network and tree algorithms, which was able to detect glaucoma with a sensitivity and specificity of more than 90%. This biomarker panel could be successfully validated in a subsequent validation study with a complete independent study population. Thus, this biomarker panel might be useful for glaucoma detection or could lead to innovative novel drug targets in future.
High Resolution Gel-based Analysis of Differentially Expressed Membrane Proteins of CD4⁺ T Helper Cells

P. Lutter¹, V. Veneruso¹, A. Dommermuth¹, J. Kubach¹, E. Schwitulla¹, H. Jonuleit², A. Wattenberg¹, S. Helling³, K. Marcus³, H. E. Meyer³, S. Muellner¹, and C. Huels¹

¹Protagen AG, Dortmund, Germany; ²Institute of Dermatology, University of Mainz, Mainz, Germany; ³Medical Proteom-Center, Ruhr-University Bochum, Bochum, Germany

CD4⁺ T helper cells are the key player for control and regulation of adaptive immune responses. In a healthy immune system they protect the body against a variety of pathogens but CD4⁺ T cells are also involved in pathogenesis and perpetuation of diverse autoimmune diseases like chronic rheumatic inflammation. Consequently, analysis of T cell activation has the potential to assist the development T cell-directed therapies and diagnostic approaches. Membrane proteins are of specific interest because they represent 60% of known drug targets and have also the potential to serve as diagnostic markers.

The membrane proteomes of a well characterized set of freshly isolated and CD3/CD28-stimulated human CD4⁺ T cells were analyzed by high resolution 2D-IEF-SDS-PAGE. Membrane proteins were enriched by differential centrifugation and following Triton-X114 extraction. Reproducibility of this isolation method for membrane proteins and 2D-PAGE was proven by comparison of proteomes of resting and activated CD4⁺ T cell samples derived from individual human donors. Using differential centrifugation and detergent extraction protein spots were displayed in proteomes that were not detectable or underrepresented in 2D gels of whole cellular lysates without any fractionation.

Furthermore, for quality control of the procedure a selection of well known surface antigens was quantitatively measured by FACS analysis prior to electrophoresis. These antigens were stained by large 2D-PAGE western blot of the separated T cell proteins identified by mass spectrometry. They were assigned to the corresponding 2D gel maps for quantitative spot analysis. Recovery as well as expression ratios of surface antigens between resting and activated T cells were compared.

A Service Oriented Architecture for Mass Spectrometry Data Analysis

G. Babnigg and C. S. Giometti

Argonne National Laboratory, Argonne, Illinois, USA

Protein identification on the basis of peptide mass and sequence information requires searching protein sequence databases with a search engine. There are several search engines available for processing tandem mass spectrometry data, including Mascot, Sequest, and X! Tandem. Each search engine requires differing parameter files and input files. In order to streamline the interpretation of tandem mass spectrometry data obtained by LCQ, LTQ, and QTOF instruments, we have developed a system where input and output files can be generated interchangeably from a common format. In addition, a common parameter file can be used for the generation of search engine-specific parameter files. Using a protein database based on unique protein sequence identifiers (Sequence Globally Unique Identifiers; SEGUID)—derived from primary protein sequences—facilitates the integration of search results. Identical copies of the species protein databases are pre-processed according to the specific search engine needs. Here we present a Service Oriented Architecture based on web services for the streamlined processing and querying of tandem mass spectrometry data. The search results are stored in the database and made available using web services for easy integration into user interfaces. The web interfaces and web services were built using .NET2.0. The web services were deployed on single servers running the Mascot or Sequest search engines. X! Tandem web services can be deployed on several machines. The use of a Service Oriented Architecture and the SEGUID database strategy allows efficient and accurate protein identifications for proteome studies by capitalizing on the information available in a broad range of protein sequence databases and differing search engine capabilities.

This work was supported by the U.S. Department of Energy, Office of Biological and Environmental Research GTL:Genomics Program under Contract No. W-31-109-ENG-38.
Antibody Screening Method Based on Microarray with Silver Enhancement Detection

H. Han¹, Z. Yu¹, Z. Liu¹, H. Du², M. Li², J. Gao¹, Q. Sun¹, and D. Xu¹

¹Beijing Proteome Research Center, Beijing, China; ²South Medical University, Guangzhou, China

With the development of genomics and proteomics, antibody microarrays are playing a more and more important role due to their powerful function of high throughput and parallel analysis. To design and fabricate an antibody microarray, it is essential to develop the screening strategy for requiring antibody properties such as their affinity and specificity. This paper presents a new analytical approach based on antibody microarray coupled with biotin labeled technique and colorimetric silver enhancement detection. In this report various antibodies and rat IgG (the negative control) and biotin-conjugated rat IgG (the positive control) were first spotted on the aldehyde-modified slide, respectively. Antigens were labeled with activated biotin and then purified by the dialysis procedure. To assay the affinity, the relevant labeled antigen was incubated with the microarray, followed by the addition of avidin-gold nanoparticle and silver enhancement reagents. The resulting colorimetric image was scanned by CCD and the gray intensity values (G) were further analyzed with software to acquire the quantitative results corresponding to each antibody spots. A threshold (Gnegative control/Gpositive control x 100 + 2SD) was introduced as a positive standard to screen and select positive antibodies. In order to obtain the specificity information as well as effective concentration qualification range of detection of these antibodies, a serial of various concentrations of antigens was reacted with the antibody microarray. The established method showed that the concentration calibration range are generally from 17.2 ng/ml to 200 ng/ml and the detection limit could approach to 0.5 ng/ml for anti-human albumin. The presented method could provide important information such as specificity and sensitivity as well as quantitative detection range for the antibodies. Compared with conventional fluorescent image detection, the colorimetric silver enhancement detection was proven to be a cost-effective and simplicity method.

Quantitative Proteomics Yields Insight into the Secretory Pathway of Rat Liver

A. Gilchrist¹, C. E. Au¹, J. Hiding², A. W. Bell¹, J. Fernandez-Rodriguez², S. Lesimple¹, H. Nagaya¹, L. Roy¹, Sara J. C. Gosline¹, M. Hallett¹, J. Paiement³, R. E. Kearney¹, T. Nilsson², and J. M. Bergeron¹

¹McGill University, Montreal, Quebec, Canada; ²Göteborg University, Göteborg, Sweden; ³Université de Montréal, Montreal, Quebec, Canada

Organelles of the early secretory pathway (rough and smooth microsomes of endoplasmic reticulum and Golgi apparatus) were isolated from rat liver parenchyma for proteomics analysis. We report the identification of more than 2,000 proteins (via 140,000 tandem mass spectra) from these subcellular fractions. Using methods of organelle isolation, biochemical subfractionation and the cell-free generation of sub-organelles (COPI vesicles) led to a near complete characterization of the proteomes of the endoplasmic reticulum and Golgi apparatus. As a measure of protein abundance, we used redundant peptide counting which we validated for this study. The use of hierarchical clustering techniques allowed for the assignment of ca 1400 proteins to their cognate organelles and ca 600 proteins to be identified as contaminants. The characterization and relative abundance of proteins in rough, smooth ER, Golgi cisternae and Golgi generated COPI vesicles provided insight into protein trafficking through these compartments. Using the redundant peptide counting approach we determined that Erp44 is Golgi located, which is contrary to current literature but validated in this study by immunolocalization. Of the 1400 proteins, some 350 have unknown functions. Three were selected for validation of their proteomics predicted location including a protein we name as sapreticulin. This study provides by far the most complete catalogue of the ER and Golgi proteomes with insight into their identity and function.
vMALDI High Energy CID, vMALDI PSD and AP-MALDI Low Energy CID of Peptides Derived from Hydrophilic and Hydrophobic Glomerular Proteins

C. Mayrhofer¹, S. Krieger², D. Kerjaschki², and G. Allmaier¹
¹Vienna University of Technology, Vienna, Austria; ²Medical University of Vienna, Vienna, Austria

Puromycin aminonucleoside (PA) nephrosis, which is characterized by severe morphological changes of podocytes, is a well established rat model closely resembling human minimal change disease. For defining changes in protein expression in association with the PA nephrosis induced impairment of the glomerular filtration barrier manifesting as proteinuria, hydrophilic and hydrophobic proteins extracted from normal and nephrotic rat glomeruli were examined by DIGE. 23 protein spots were found to be differentially expressed in response to PA. Now MS based sequencing of the enzymatic generated peptides thereof was used to obtain specific sequence tags allowing the unambiguous identification.

In the present study, two types of desorption/ionization techniques combined with different modes of CID, namely vacuum matrix-assisted laser desorption/ionization (vMALDI) high energy CID and post-source decay (PSD) as well as atmospheric pressure (AP-) MALDI low energy CID, were applied for the fragmentation of singly protonated peptide ions, which were derived from 2D-separated, silver-stained and trypsin-digested hydrophilic as well as hydrophobic glomerular proteins. Thereby, defined properties of the individual fragmentation pattern generated by the specified modes could be observed. Furthermore, the compatibility of the varying PSD and CID (MS/MS) data with database search derived identification using two public accessible search algorithms has been evaluated. The peptide sequence tag information obtained by PSD and high energy CID enabled in the majority of cases an unambiguous identification. In contrast, part of the data obtained by low energy CID were not assignable using similar search parameters and therefore no clear results were obtainable. The knowledge of the properties of available MALDI based fragmentation techniques presents an important factor for data interpretation using public accessible search algorithms and moreover for the identification of 2D-gel separated glomerular proteins.

LC-MALDI Top-Down Biomarker Profiling and Identification

D. Suckau, S. Brand, S. Hahner, A. Asperger, and M. Meyer
Bruker Daltonics, Bremen, Germany

The search for new and validated biomarkers is of particular interest in different clinical areas like oncology, neurology, toxicology and pharmacology. One of the challenges in finding the right technology for biomarker research is to combine a statistical reasonable throughput—hundreds of samples—with an in-depth proteome technology. As proteolytic events play a significant role, in particular in disease related events, biomarker discovery approaches may benefit from a top-down profiling approach, as proteolytic isoforms remain intact during the analysis.

We combine serum sample preparation based on magnetic nanoparticles purification (WCX) or other methods with the high resolution HPLC-MALDI analysis of the undigested peptides and proteins. Proof of principle experiments included 20 serum samples vs. 20 serum samples spiked with peptides and proteins <10 kDa.

Multivariate statistics (PCA) detected the spiked materials correctly from the complex matrix and subsequent MS/MS spectra allowed their identification. First experiments clearly demonstrate that this technology significantly increases the number of detectable signals received from human serum (>1500) and is very reproducible on the 10 sec fraction size level. Therefore this approach opens the door for high throughput in-depth analysis of clinical samples for the detection of biomarkers. Furthermore the reduction of protein complexity per fraction after LC-MALDI separation enables using a simple and fast method for the identification of biomarker candidates: in situ digestion provides for protein identification directly from LC runs collected on MALDI targets. Thus we close the gap between detection of biomarker candidates and their final identification. This ID is mandatory for their validation and for any further diagnostic use of biomarkers.
Free-Flow-Electrophoresis, an Efficient Method for High Purity Preparations of Organelles

A. Aboldzade-Bavil, M. Nissum, R. Wildgruber, M. Islinger, H. Mohr, and C. Eckerskorn

1BD Diagnostics, Planegg, Germany; 2Institute of Anatomy, University of Heidelberg, Heidelberg, Germany

The preparative dissection of cells into their substructure reduces sample complexity and facilitates functional analysis of proteins in a physiological context. Typical methods for separating organelles from other cellular components result in insufficient purity by cross contamination of other sub-cellular components and require ultracentrifugation steps. We have developed a protocol for the fractionation of subcellular organelles using BD™ Free-Flow-Electrophoresis (FFE) System that does not include ultracentrifugation. A low degree of cross-contamination was observed using this procedure.

In this FFE methodology, particles are deflected differently within an electric field, according to their net surface charge and size, and are separated through a buffer flow perpendicular to the electric field. With this technique, successful isolations of various cellular structures have been obtained. For organelle separation from eukaryote samples (e.g. animal tissue and yeast), standard serial procedures have been developed. After FFE protocols, subcellular fractions were separated by SDS-PAGE, and visualized and analyzed in more detail by immunoblotting using organelle specific antibodies. The respective subcellular fractions were also assayed for the activity of the indicated marker enzymes.

Electron microscopy of the organelle fractions extracted using the FFE system indicates that the purification was selective on the organelle level. Purity is also confirmed by multiple tests on the protein level: 1) immunoblotting with antibodies directed against appropriate organelle-specific marker proteins, 2) the protein patterns of the respective fractions are clearly distinct, and 3) activity assays for organelle-specific enzymes, which also demonstrates that FFE separation conditions also leave enzymes in their native, functional states.

Differential Protein Expression during TGFβ-Induced Epithelial-Mesenchymal Transition in Lung Cancer Cells Utilizing the SILAC Strategy

R. Amunugama, V. Keshamouni, D. Allen, R. Hagler, G. Omenn, A. Nesvizhskii, and M. Pisano

1Proteomic Research Services, Inc, Ann Arbor, Michigan, USA; 2University of Michigan, Ann Arbor, Michigan, USA

In cells with intact TGFβ signaling, TGFβ promotes tumor progression and metastasis by its pleiotropic activities on both cancer cells and surrounding stroma. In several epithelial tumors, TGFβ reactivates a fundamental process known as the epithelial to mesenchymal transition (EMT) that governs morphogenesis in multicellular organisms. During EMT, epithelial cells acquire fibroblastoid morphology, down-regulate epithelial-specific proteins, express mesenchymal-cell markers, and acquire a migratory/invasive phenotype. There is good evidence that EMT leads to metastasis.

Treatment of A549 lung adenocarcinoma cells with TGFβ induces EMT and results in a dramatic change in cell morphology, acquiring migratory and invasive phenotypes. To gain insights into molecular events associated with EMT we employed Stable Isotope Labeling of Amino acids in Cell culture (SILAC), followed by iso-electric focusing, SDS-PAGE and LC-MS/MS (LTQ) analysis, for quantitatively identifying differentially expressed proteins during EMT. In our analysis we identified the mesenchymal-marker protein vimentin as up-regulated and the epithelial-marker proteins cytokeratin 1 and 9 as down-regulated, consistent with the process of EMT. We also identified several proteins with increased expression (filamins, gelsolin, microtubule-associated protein, tropomyosins, HSP27, calpain) that reflect the extensive cytoskeletal reorganization that occurs during acquisition of fibroblast morphology and associated cell motility.

We anticipate that this complete analysis will identify additional novel protein regulators of EMT that may serve as potential therapeutic targets against metastasis. [Supported by MTTC grant 687]
Central nervous system hemangioblastoma (CNS HB) usually locates in posterior skull fossa, accounts for 0.9–3.5% of intracranial tumors. The details of the formation and proliferation are still not fully understood till now and the origin of its histology is much disputed. Since protein is the genuine executor of the life, it is important to investigate the overall proteins of HB systematically in order to gain more insight into the role of CNS HB.

We have investigated in the first the concrete mechanism of CNS HB using proteomics technique. We applied two-dimensional gel-electrophoresis in combination with mass spectrometry for its powerful approach to compare protein expression in brain tissues. By comparing the 2-D gel maps of HB with normal brain tissue, 51 discrepant protein spots were identified. With the experimental results, we have established detailed protein expression characteristics of HB, and found out the difference between HB and normal brain tissues in protein expressions that may be significantly associated with HB occurring.

We have confirmed that vimentin and 14-3-3 proteins play an important role in the HB occurring. The results of the immunohistochemistry staining of vimentin and 14-3-3 proteins in the sections of HB and normal brain tissues, also explored that two proteins were distinct positive in HB section and were negative in normal brain tissues section. Considering the benign and tumor-like growing characteristic of CNS HB, we conclude that CNS HB may be a tumor of central nervous system which roots in normal brain tissues (parenchyma or mesenchyma). The process of HB occurring is an intricate, multicomponent, multifactorial and multi-step process which is mediated by a variety of proteins. CNS HB may be a kind of tumors of central nervous system, come from the brain mesenchymal tissues, and possibly root in stem cells.

Free-flow Electrophoresis, a Solution-based Orthogonal Separation Dimension in the Proteomics Workflow

M. Nissum, R. Wildgruber, G. Weber, and C. Eckerskorn
BD Diagnostics, Planegg, Germany

Progress in proteome analysis relies heavily on the development of technologies to reduce the complexity of the sample prior to the application of analytical techniques like gel electrophoresis, liquid chromatography and mass spectrometry. Free-Flow-Electrophoresis (FFE) offers a powerful approach to the separation of charged species including peptides, proteins, organelles and cells. Due to the matrix-free fractionation and therefore the absence of any solid phase interactions, FFE offers advantages over traditional chromatographic and gel-based techniques: separation from small molecules like peptides to particles like organelles, native separation conditions, high sample recovery, fast fractionation times and high throughput.

The traditional way of reducing complexity in shotgun proteomics includes strong-cation-exchange chromatography in the first separation dimension followed by reversed-phase (RP) chromatography. The peak capacity of such a system is limited by the separation principles of the two methods, which, in this approach, are not independent. Introduction of isoelectric focusing as a first dimension provides a truly orthogonal separation dimension to pair with RP chromatography. We have performed IEF of peptides in solution using FFE. The matrix-free separation medium increases sample recovery and is compatible with subsequent chromatographic separation. The FFE methodology was applied to the plasma proteome and compared to the similar approach on the protein level.

In order to demonstrate the versatility of the FFE methodology, we have separated a wide range of complex samples. Each of these samples represents sub-populations of entire proteomes, such as membrane proteins or cell lysates. High FFE resolution is demonstrated using a mixture of protein isoforms, for which thorough separation requires many fractions across a narrow pH range. In addition, the FFE methodology was shown to be highly compatible with not only plasma but also other body fluids such as urine, saliva and cerebrospinal fluid.
Rat Liver Detoxification Machinery as Determined by Proteomics Analysis of Enriched Rough and Smooth Microsomes and Golgi Fractions

A. W. Bell¹, S. Lesimple¹, P. C. Boutros², S. J. C. Gosline¹, C. Au¹, A. Gilchrist¹, J. Paiement², R. E. Kearney¹, A. B. Okey², M. T. Hallett¹, and J. J. M. Bergeron¹

¹McGill University, Montreal, Quebec, Canada; ²University of Toronto, Toronto, Canada; ³Universite de Montreal, Montreal, Quebec, Canada

Proteomics analysis (LC-MS) of 1D-SDS PAGE, in-gel trypsin digested highly enriched preparations of rough and smooth microsomes and Golgi apparatus isolated from male rat liver homogenates has identified 120 proteins that are involved in lipid oxidation, steroid metabolism and drug detoxification. Quantification was by redundant peptide counting (spectral counts). Further enrichment was achieved by biochemical fractionation employing high salt wash and Triton X-114 detergent phase extraction, confirming the lumen/membrane location and revealing an additional 17 proteins involved in lipid oxidation, steroid metabolism, and detoxification. The most abundant detoxification proteins identified were from the multi-gene families of the cytochrome P450, glucuronosyl transferase and carboxylesterase proteins, with 35, 11 and 9 members identified, respectively. Together with the cytochrome P450 and cytochrome b5 reductases, this represents one of the most extensive proteomics characterizations of the detoxification machinery in rat liver. Comparison with transcriptomics data has been made. Of a subset of 79 proteins characterized by proteomics for detoxification, only 43 were found in the transcriptome. For these 43, a subset showed a positive correlation but with the majority not correlated and likely influenced by post-transcriptional events. Hierarchical clustering of the proteomics data indicates trafficking of a subset of the proteins giving insight into the mechanism for retention of the proteins in domains of the endoplasmic reticulum.

Analytical Performance Characterization of an FT-MS-based Label-free Platform for Biomarker Discovery in Plasma; Standards, Precision, Accuracy, and Directed Feature Investigation

J. N. Sutton¹, M. Athanas², T. Richmond¹, X. Shi³, A. Zumwalt¹, R. E. Gerszten³, and L. E. Bonilla¹

¹Thermo BRIMS Center, Cambridge, Massachusetts, USA; ²The BioTeam, Cambridge, Massachusetts, USA; ³Massachusetts General Hospital, Boston, Massachusetts, USA

In this report we present a detailed methodological and instrumental description of an FT-MS-based, label-free platform for biomarker discovery in human plasma. We will discuss the relevant analytical figures of merit for each of the main steps of this workflow, namely immunoaffinity depletion of the top-12 most abundant plasma proteins; high-precision and high-resolution LC-MS/MS analysis using a unique split-flow design; robust differential MS signal analysis using a robust high-throughput computational assembly that employs new chromatographic alignment and statistics-based differential expression algorithms, followed by directed feature (frame) identification using SEQUEST. The application of this analytical platform to a relevant clinical model will be discussed, as well as its extension to targeted feature analysis by complementary MS strategies (ECD, IRMPD), and potential new applications like metabolomics.
New Developments in MALDI Imaging Mass Spectrometry for Pathological Proteomic Studies; Introduction to a Novel Concept, the Specific MALDI Imaging


1MALDI Imaging Team FRE CNRS 2933, Lille, France; 2EUROGENTEC, Liege, Belgium; 3Jeanne de Flandres Hospital, Lille, France

MALDI direct analysis and MALDI imaging of tissues have shown to be a very powerful tool by localizing molecules in tissue and by avoiding extraction, pre-purification, separation. Nevertheless, this new method require new developments to increase sensitivity and specificity.

We propose here a new concept and evolutions of each step of MALDI Imaging analysis. New matrices called Ionic matrices were synthesized to be especially well adapted for a good crystallization, sensitivity and resolution compared to the classical matrices HCCA or SA. Moreover, in order to work on tissue kept in hospital libraries, FFPE tissues (Formalin Fixation and Paraffin Embedded) were studied. So as to avoid such problems another reactive matrix has been developed. But, tissue fixation raises many problems for mass spectrometry (MS) analysis especially for long stored tissues. (>1 year) Though, a new strategy of in situ tissue enzymatic digestion was developed in combination with automatic micro-spotting MALDI Imaging.

In order to add a dimension of specificity to imaging by MS, we have developed specific imaging using designed probes directed against specific targets with mass spectrometry detection. This strategy has been developed for different class of biomolecules with a specific highlight, to mRNA and peptides/proteins. This concept is based of indirect detection of specific targeted molecule using a photocleavable tagged probe. The introduction of the specific imaging give another dimension by targeting specific disease-marker-gene RNA transcripts, following their expression within tissues and then confirming their translation by targeting their specific protein products or metabolites. Disease/health states will thus be closely molecularly monitored.

Application of MALDI profiling and imaging were studied on ovarian cancer case. By looking to peptides content modifications from compared biopsies of benign versus cancer patients, several potential biomarkers were identified and validated.

Taken together, Specific MALDI Imaging open the door of a new fields of research.

A Cost-effective Protein Array Fabrication and Detection Technique Based on the Microcontact Printing and Silver Enhancement Method

X. Yu, Z. Liu, W. He, W. Liao, and D. Xu

Beijing Proteome Research Center, Beijing, China

In recent years, protein microarray has played a more and more important role in the clinical diagnostic and proteomics owning to assaying biological molecular interactions in large scale, high-throughout and high-sensitivities. Just as deriving from ELISA and gene microarray, many protein microarray fabrication and detection techniques are highly automatic now, such as pin arrayer printing robots or ink-jet printers and fluorescent scanner etc. However, the high-cost of these automatic instruments limits their applications popularly.

In this study, we firstly develop a set of cost-effective and sensitive protein array fabrication and detection technique by combining the agarose stamper with silver enhancement method. At the beginning, the homogeneity of the spots on the protein array and quantitative transferring capability of the agarose stamper as well as the activities of transferred proteins were checked by the fluorescent method. Then direct and sandwich reaction strategies were employed to test the ability of quantitative analysis of this conjugation technique, respectively. The results demonstrated that the proteins can be transferred to the slide quantitatively and homogeneously (CV <10%). Moreover, the detection limit of 0.33 fmol for direct direction and 0.13 fmol for sandwich detection were obtained, which are comparable with that of fluorescent method. Finally, two indexes were introduced to evaluate the effective number of repetitive stamping using the patterned agarose stamper, and we found that seven protein arrays can be prepared in one time without intermediate re-inking. With this conjugation technique, a lot of cost can be saved, which is very helpful for the popularity of protein array. Moreover, its versatile ability of fabricating protein array and high sensitivity holds its promising to be employed in the research of clinical diagnostic as well as proteomics.
B-PEG Films for Desalting-Free and Enrichment of Salt-contaminated Peptide Sample in MALDI-TOFMS

H. Lu¹, J. Fang², and P. Yang³

¹Department of Chemistry, Fudan University, Shanghai, China; ²Jiaotong University, Shanghai, China; ³Institutes of Biomedical Sciences, Fudan University, Shanghai, China

MALDI-MS has encountered the challenge to identify low abundance proteins with contaminants of salts. Sample cleanup procedure, therefore, must be performed prior to analysis. However, all the previous desalting techniques depended on the stronger interaction force between analytes and stationary phase, and on the weaker force between contaminants and stationary phase. All these conventional methods need a washing step that is time-consuming and in the same time result in sample loss.

We developed an intelligent method to achieve peptides enrichment and desalting with no wash step. The polymer B-PEG was firstly dissolved in THF, then polymer solution was spotted on the target, a homogeneous film could form after solvent evaporated. Subsequently, the sample can be directly analyzed by MALDI-MS after the sample solution was spotted and dried on the B-PEG film, in the presence of contaminant of ammonium bicarbonate (ABC), SDS, NaHCO₃ or CHAPS.

It is founded that the B-PEG film exhibits some remarkable features in salts adsorption and peptides enrichment. The first advantage is that the B-PEG film can strongly adsorb salts. And second, the enrichment of peptides could be completed simultaneously and sample loss is avoided due to no washing step. The third but not last, the polymer film as substrate results in a good reproducibility of signals. The digestion peptides of myoglobin with concentration of 30 fmol/µL containing 100 mM ABC could hardly be detected on untreated target. However, using the B-PEG strategy, the S/N of the most three strongest peaks were increased from 20 to 680 (m/z 748.45), 10 to 180 (1271.69) and 33 to 240 (1606.88), respectively. The proteins extracted from the liver of house-mouse were separated by 2DE and 30 protein spots were analyzed. The B-PEG strategy has been proved to be effective in this real proteomic analysis.

A Comparative Study of iTRAQ- and ICPL-based Protein Quantification


Bruker Daltonics, Bremen, Germany

Experiments based on Stable Isotopic Labeling and LC-MS/MS measurement techniques attract more and more attention in Quantitative Proteomics. They are increasingly applied in studies of post translational modifications (PTMs), and biomarker discovery as they provide broad proteome coverage and increased statistical relevance of quantitative results.

We describe a software platform which supports all popular labeling strategies like ICAT™, ICPL™, iTRAQ™, ¹⁸O/¹⁶O, and SILAC™ in combination with a variety of different types of mass spectrometers, like MALDI-TOF/TOF, ESI-IonTrap, ESI-(Q)-TOF and Qh-FTMS. The software platform is especially suited for data validation as it provides sophisticated visualizations tools for all kinds of data gathered during the experimental workflow. Automatic outlier detection is based on median statistics and manual validation work can benefit from simultaneously available average and median statistical data. We used the platform in the development of applications for a novel triplexed quantification technology and for a comparative study on iTRAQ and ICPL.

iTRAQ (ABI), an isobaric peptide label technology where quantification is based on MS/MS reporter ions and ICPL (Bruker) as non-isobaric protein label were applied to model protein mixtures (14 proteins) and analyzed either on a MALDI-TOF/TOF or an ESI-qTOF using the same software platform WARP-LC 1.1 (Bruker). The analysis resulted in < 10% error margins for ICPL and details of the optimized data handling will be discussed.
Prespotted AnchorChip Targets as Archiving Platform for Proteomics Samples

C. Luebbert¹, C. Ziegmann², M. Schuerenberg¹, and D. Suckau¹
¹Bruker Daltonics, Bremen, Germany; ²Eppendorf Polymere GmbH, Oldenburg, Germany

Archiving of MALDI sample preparations is an important issue whenever there is a significant time lag between sample preparation and mass spectrometric analysis, one has to revisit selected samples under different aspects, or simply in order to verify results. Any kind of long-term archiving requires a cost-effective, reliable platform technology and dedicated storage conditions. We used so-called “prespotted AnchorChip” targets for archiving proteomics samples under different storage conditions.

92 Coomassie stained gel spots and an LC-MALDI run containing 384 fractions were archived on prespotted AnchorChip targets (Bruker) for one month at room temperature under nitrogen. For these real-life samples the archiving-related information loss was studied based on 500 MS and more than 2000 MSMS spectra acquired on a MALDI-TOF/TOF instrument.

In addition, 250 amol and 5 fmol tryptic BSA digest were archived on the same type of target for up to one year. Six different archiving conditions were tested: vacuum vs. air vs. nitrogen at different temperatures; 20°C, 4°C or −18°C. Sample quality was determined by MS and MS/MS sequence coverage, Mascot score and number of matching peptides. The BSA model digest experiments have shown that even 250 amol can safely be identified after one year storage at room temperature in a sealed plastic bag. Above 1 fmol samples can be archived for some months at room temperature in a sealed plastic bag without significant loss. Archiving the targets in a freezer slightly improves the results for longer storage periods or for sub-fmol samples. MS and MSMS identification rate of in-gel digest remained unchanged during the 4 weeks archiving period.

Archiving of LC-MALDI runs opens the doors for many profiling applications in which MS/MS acquisitions may be intelligently triggered weeks or months after the initial MS analysis has taken place.

The Application of Orthogonal Fractionation Techniques to Reduce Sample Complexity for Proteomic Studies

J. Smith, D. Kalman, and H.-C. Tran
Alfa Wassermann, West Caldwell, New Jersey, USA

Current protein expression profiling technologies are challenged to provide a comprehensive analysis of complex proteomes. Sample fractionation is essential to overcome these problems by reducing sample complexity. Multi-dimensional fractionation and separation techniques can further simplify sample complexity making more proteins accessible for detection and thereby expanding protein profiling capabilities. As a result, it is possible to identify a greater number of low-abundance proteins from a complex protein mixture. In this study, the AW Promatix 1000™ was used to enrich and concentrate organelles and subcellular particles, in a non-denaturing manner, based on their buoyant densities. Homogenized rat liver was centrifuged at low speed to remove nuclei and the resulting post nuclear supernatant (PNS) was fractionated on an AW Promatix 1000™ instrument. The AW Promatix 1000™ provided a level of sample simplification by providing enriched organelles including intact mitochondria. Samples fractionated on the AW Promatix 1000™ were further simplified using an orthogonal technique, solution phase IEF, to reduce sample complexity even more. After two-dimensional separation, fractions were subjected to two-dimensional gel electrophoresis (2DE) for protein separation and identification. 2DE gels revealed the unique benefits provided by the AW Promatix 1000™ and showed that it can be combined with other techniques to reveal a complex proteome. Over 40 proteins that were previously undetected were identified after multi-dimensional separation. Reduction of sample complexity through organelle isolation is shown to facilitate the identification of low abundance proteins.

Reduced Subjectivity in 2D-PAGE Analysis Using SameSpots

D. Bramwell, I. Reah, D. Miller, and A. Kapferer
Nonlinear Dynamics, Newcastle, United Kingdom

2D-PAGE experiments are commonly used to highlight significant changes in different experimental conditions, such as control-treated, diseased-undiseased, or to investigate interesting protein expression behaviour across, for example, a time or dosage series.

Traditionally, a large amount of post-detection editing has been required in order to prepare the data for statistical analysis. This process, however, is user-dependent delivering highly variant, subjective results. With the introduction of SameSpots, 100% matching with no missing values is achieved without the need for editing. This results in an increased statistical power and also reduces subjectivity by employing a standardised workflow, containing high levels of automation, minimising user interaction.

Using a DiGE experiment as a template (18 images, 2 classes) we had 5 users of varying experience perform the analysis using the SameSpots workflow. One user was an expert user and their results used as the control. No editing was performed or required. The list of spots from each user was ordered by statistical significance.

All of the expert user’s top 10 spots appeared in the other users’ top 25. At least 70% of the expert’s top 10 spots were represented in each other users’ top 10.

Using the SameSpots approach improves the objectivity and repeatability of 2D experimental analysis, increase throughput with a fast analysis requiring no time-consuming editing, and increase the statistical power of the data through 100% matching and no missing values.
Effect of Iron Availability on Protein Profiles of Human Pathogen *Trichomonas vaginalis*  
J. B. De Jesus¹, P. Cuervo¹, M. Junqueira³, F. C. Silva-Filho², C. Britto¹, E. Cupolillo¹, O. Fernandes¹, and G. Domont³  
¹IOC/FIOCRUZ, Rio de Janeiro, Brazil; ²IBCCF/UFRJ, Rio de Janeiro, Brazil; ³IQ/UFRJ, Rio de Janeiro, Brazil

*Trichomonas vaginalis* is the causative agent of human trichomoniases, the most frequent non-viral STD accounting for approximately 200 million annual cases world-wide. This parasite is emerging as an important co-factor in amplifying HIV transmission and it is associated to cervical cancer, atypical pelvic inflammatory disease and infertility. In pregnant patients infection has been associated with pre-term delivery and low-birth-weight. It has been suggested that the mechanisms by which *T. vaginalis* causes these alterations are influenced by iron. This metal modulates adhesin synthesis, resistance to complement lysis, binding to extracellular-matrix proteins and expression of proteases of *T. vaginalis*.

The aim of this work was to identify the changes induced in the proteome of *T. vaginalis* when this protozoa is grown in iron-depleted medium, using the combination of two-dimensional gel electrophoresis and mass spectrometry. Parasites grown in iron-rich and iron-depleted medium were lysed by freeze/thawing and proteins were precipitated with TCA and cold acetone wash. Pellets were dissolved in 9M Urea, 4% CHAPS, 60mM DTT and 0.2% ampholytes. Protein profiles were obtained in strips of pH 4–7 and 12% polyacrylamide gels. Peptides from in gel tryptic hydrolysis were collected, submitted to MS and MS/MS analysis and searched at NCBI using MASCOT software. From 43 proteins differentially expressed identified by MS/MS in parasites grown in iron depleted medium, 8 proteins were overexpressed and 32 were repressed. In addition, 2 proteins were exclusively observed in gels derived from parasites grown in iron-depleted medium. Identified proteins belong to distinct functional groups as cytoskeleton, glycolytic pathway, heat shock, amino acid metabolism and anti-oxidative stress.

Peptide-based Sandwich Immunoassays, a Novel Approach to Quantify Phospho-Proteins  
O. Poetz¹, T. Nadler², J. Boucher², N. Gordon², S. Pabst¹, D. Stoll¹, M. Templin¹, and T. Joos¹  
¹NMI Natural and Medical Sciences Institute, Reutlingen, Germany; ²Epitome Biosystems, Waltham, Massachusetts, USA

Since their introduction by Berson and Yalow quantitative immunoassays (Yalow and Berson, 1960) developed into a key technology for the life sciences. Proteinarrays that miniaturize and parallelize immunoassays allow highly sensitive and quantitative measurements for a higher number of proteins in one reaction (Poetz et al., 2005; Templin et al., 2003). The bottleneck for a quantitative determination of protein content and of post-translationally modified variants is the availability of standard proteins. Here mass spectrometry based approaches that utilise synthetic peptides as standards for quantitation of proteins are in use.

Like in mass spectrometry based approaches, our novel method utilizes peptides as analyte and as standards for quantitative measurements of proteins and phospho-proteins, but peptides are used in the highly sensitive sandwich immunoassay format. Synthetic signature peptides (Epi-Tag TM) characteristic for analyte proteins are synthesized and serve as easily accessible antigens for the generation of peptide specific antibodies. These antibodies are utilized as capture and detection reagents in protein arrays. Proteolytically digested cells or tissue serve as samples and the content of the proteins can be calculated from peptide standard curves. The assays have been adapted to a bead based multiplexed immunoassay format (Luminex 100 TM). This allows the simultaneous identification and quantification of proteins and phospho-proteins based on their proteolytical fragment.

The characteristics of such a signature peptide based immunoassay is shown for EGF-stimulated cell lines. We show that even a low amount of absolute levels of ERK and phosphorylated ERK can be measured and time courses during EGF-stimulation can be easily obtained. The advantages and the potential applications of our approach will be discussed.
A Novel Approach to 2D-PAGE Analysis Using the SameSpots Workflow

D. Bramwell, I. Reah, D. Miller, and A. Kapferer
Nonlinear Dynamics, Newcastle, United Kingdom

2D-PAGE experiments are commonly used to highlight significant changes in different experimental conditions, such as control-treated, diseased-undiseased, or to investigate interesting protein expression behaviour across, for example, a time or dosage series.

The analysis of 2D experiments typically comprises a lengthy setup and computational stage, followed by a large amount of post-detection editing in order to prepare the data for statistical analysis. Here we introduce the SameSpots workflow which simplifies and automates experimental analysis, reducing average time spent per gel and so increasing throughput. The SameSpots workflow comprises advanced, semi-automatic image alignment technologies; followed by feature detection using a normalised, statistical model of the entire experiment. Post-analysis normalisation and statistical ordering are performed automatically using standardised techniques, reducing the subjectivity of results.

Workflow overview: Semi-automatic image alignment; Automatic feature detection; Automatic application of SameSpots across the experiment; Manual experimental class definition; Automatic statistical spot ordering; Manual spot review and selection; Automatic report generation.

An example run took just over 2 hours from experiment set up to final results report. An average of 6 minutes per gel compared to the typical quoted time of 8 hours per gel. This analysis was performed on a non-DIGE experiment with 2 classes and 10 replicates per class.

Using the SameSpots approach improves the objectivity and repeatability of 2D experimental analysis, increase throughput with a fast analysis requiring no time-consuming editing, and increase the statistical power of the data through 100% matching and no missing values.